

A teaching laboratory on the activation of xenobiotic transporters at fertilization of sea urchins

18

Lauren E. Shipp^{*,1}, Rose Z. Hill[†], Amro Hamdoun^{*}

^{*}*Marine Biology Research Division, Scripps Institution of Oceanography, University of California,
San Diego, La Jolla, CA, United States*

[†]*Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA,
United States*

¹*Corresponding author: e-mail address: lshipp@ucsd.edu*

CHAPTER OUTLINE

1 Introduction.....	430
2 Laboratory Day 1: Activation of Transporters at Fertilization and the EPA Toxicity Test... 432	
2.1 Background.....	432
2.2 Specific Activities and Learning Goals.....	435
2.3 Materials.....	436
2.4 Part A: Visualizing Embryonic Protection Using a Chemical Efflux Assay and Fluorescence Microscopy.....	436
2.4.1 Procedure.....	436
2.4.2 Questions.....	438
2.5 Part B: the Sea Urchin Fertilization EPA Toxicity Test.....	439
2.5.1 Procedure.....	439
2.5.2 Questions.....	440
3 Laboratory Day 2: Analyzing and Presenting Scientific Data.....	440
3.1 Background.....	440
3.2 Specific Activities and Learning Goals.....	441
3.3 Materials.....	441
3.4 Using ImageJ to Analyze Efflux Activity Data Collected on Day 1.....	441
3.4.1 Procedure.....	441
3.4.2 Questions.....	446
References.....	446

Abstract

Sea urchin gametes have been historically used to demonstrate fertilization and early development in student laboratories. Large amounts of egg and sperm are easily acquired, and the conspicuous changes in egg surface morphology, indicative of sperm-egg fusion and egg activation, are readily observed in the classroom. However, less often incorporated into teaching labs are exercises that demonstrate the dramatic metabolic changes that accompany egg activation. One example is the massive up-regulation of various essential transport activities in the embryo's plasma membrane, including xenobiotic transporter activity. Here we outline a laboratory that incorporates this concept into a teaching lab, capitalizing on the magnitude and uniformity of the xenobiotic transporter activation event in certain species of sea urchins. The introduction of this chapter provides background information for the instructor, and the remainder serves as a laboratory manual for students. The experiments detailed within the manual can be completed in a total of 4–8 h spread over one or two lab periods. The lab manual guides students through a modified version of the United States Environmental Protection Agency (EPA) toxicity test, a novel undergraduate-level laboratory on xenobiotic transporters, and analysis of microscope data using ImageJ. We have found this lab to be of interest to a wide range of biology and environmental science undergraduates, and effective in teaching underlying concepts in developmental biology, physiology and toxicology.

1 INTRODUCTION

The sea urchin has long been used to teach university and high-school students about fertilization and early development. Sea urchins are exceptional teaching tools in developmental and cell biology for several reasons. Their gametes are easily and inexpensively obtained in large quantities, eggs and embryos are of sufficient size and clarity to be viewed under any standard light microscope, early development is synchronous, and different species of sea urchins are available worldwide and have broad reproductive periods (Pearse & Cameron, 1991). For decades, sea urchin fertilization has been a favorite classroom activity because it occurs externally in seawater and can be observed in real-time by monitoring the formation of the fertilization envelope (FE).

In all known organisms, the fusion of egg and sperm results in massive structural and functional reorganization of the egg plasma membrane. In sea urchins, the structural changes include the fusion and release of egg cortical granules to create the FE within seconds-to-minutes of sperm and egg fusion. Subsequent waves of exocytic events lead to deposition and formation of a complex network of extracellular matrix molecules (Matese, Black, & McClay, 1997). The functional changes include a dramatic modification of egg plasma membrane (PM) selectivity toward ions and small molecules, such as nucleosides (Piatigorsky & Whiteley, 1965), phosphate (Whiteley & Chambers, 1966), amino acids (Epel, 1972), and xenobiotics (Hamdoun, Cherr, Roepke, & Epel, 2004). This is mediated by the insertion of various transporters into the PM following sperm and egg fusion (Epel, 1975).

Previous protocols have taken advantage of the sea urchin to introduce students to the structural events that accompany fertilization (Epel, Vacquier, Peeler, Miller, & Patton, 2004; Vacquier, 2011). Here we build upon these by providing a lab exercise that demonstrates the physiological consequences of membrane restructuring in sea urchin embryos. The functional change we focus on is the dramatic increase in xenobiotic transporter activity that happens after fertilization. This event can be observed in live embryos requiring only non-toxic and inexpensive dyes, a fluorescence microscope, and just a few hours to conduct the assay (Hamdoun et al., 2004).

This chapter outlines an undergraduate student laboratory on xenobiotic transport in sea urchin embryos. Because this lab addresses concepts in developmental biology, medicine, and environmental toxicology, the protocol is of interest to a wide range of biological or environmental science students. For instance, the biology of fertilization is itself a core topic in most developmental biology laboratories. In addition, the xenobiotic transporter activity that students will observe is mediated by a subset of ATP-binding cassette (ABC) transporters, proteins that may be familiar to pre-medical students due to their clinical role in drug disposition and resistance (e.g., chemotherapeutic resistance, Giacomini et al., 2010; Gottesman, Fojo, & Bates, 2002). Finally, this protocol introduces core concepts in environmental toxicology since, in addition to effluxing drugs, xenobiotic transporters also protect cells from man-made environmental toxicants. As such, the exercise is useful in illustrating the “elimination” component of ADME (Absorption, Distribution, Metabolism, and Elimination). Finally in pollution monitoring worldwide, sea urchins are a popular system (see Chapter “Methods for toxicology studies in echinoderm embryos and larvae” by Torres-Duarte et al. of this book), and this lab presents a modified version of the fertilization assay used by the United States Environmental Protection Agency (EPA) (Dinnel, Link, Stober, Letourneau, & Roberts, 1989; U.S. Environmental Protection Agency, 2002, 2009).

The exercise presented here capitalizes on two robust assays to create a novel laboratory experience with unique learning opportunities. Students will first observe the efflux-transport mechanism that protects embryos from xenobiotics. They will then measure a consequence of xenobiotic accumulation in gametes, by testing the effects of pollutants on fertilization. Finally, students will analyze and statistically evaluate their data. These activities introduce techniques that are ubiquitous in modern biology research, including conducting a fluorescence-based reporter assay, organizing electronic and tabular data, performing statistical tests, and using ImageJ freeware—which is now a requisite skill in microscopy (Schindelin, Rueden, Hiner, & Eliceiri, 2015). Upon completing the exercises, students will have gained experience with pollution monitoring methods, quantitative microscopy, and critical evaluation of data and assay design. This inquiry-driven learning experience provides an engaging way to teach principles of fertilization, membrane reorganization, transport activity, and cellular protection.

The activity is designed as a 2-day laboratory that will take 2–4 h each day, and it could alternatively be completed in a single longer period. Apart from this Introduction, the chapter is formatted such that it can be copied with little or no modification

into student laboratory manuals. Additional notes to instructors, including catalog numbers for materials, are included as chapter footnotes. The entire protocol can be completed at room temperature with gametes and embryos of *Lytechinus pictus*, and it has the potential to be modified for use with other regional species^a. The exercises are appropriate for upper division US university students—which is typically third and fourth year—but could be adapted for levels ranging from advanced high school to new graduate students. Most will benefit from having previously completed introductory (lower division) biology coursework, including at least one laboratory. While it is useful for students to conduct a sea urchin fertilization lab (Epel et al., 2004; Vacquier, 2011) prior to completing this one, it is not a requirement.

2 LABORATORY DAY 1: ACTIVATION OF TRANSPORTERS AT FERTILIZATION AND THE EPA TOXICITY TEST

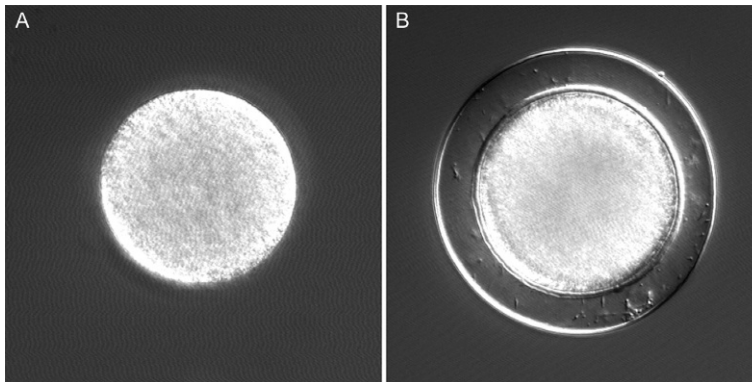
2.1 BACKGROUND

Sea urchins provide a useful model system for studying the biological effects of toxins (Chapter “[Methods for toxicology studies in echinoderm embryos and larvae](#)” by Torres-Duarte et al., this volume). Sea urchin gametes (sperm and eggs) are released into the open ocean where embryonic development proceed among many environmental challenges. To cope with these temperature, salinity, and chemical stressors, marine embryos have evolved extensive cellular protective mechanisms. In today’s industrial world however, a marine embryo’s environment contains many new, anthropogenic chemical stressors (xenobiotics) that may or may not overcome the organism’s evolved protective capabilities.

The United States Environmental Protection Agency (EPA) is a federal organization whose purpose is to regulate and enforce laws that protect human and environmental health. The EPA uses a variety of model organisms and toxicity assays to evaluate the safety of environmental contaminants and prioritize their regulation. To test the toxicity of effluents discharged into the environment, the EPA conducts a fertilization assay using sea urchin gametes (U.S. Environmental Protection Agency, 2002, 2009).

Sea urchins are an optimal system in which to assay fertilization because the merging of egg and sperm is accompanied by the appearance of a fertilization envelope (FE, Fig. 1), and the presence of the FE can be easily and rapidly scored under a low-power microscope. FE formation occurs in response to a rise in intracellular

^aTo allow the experiments to be conducted at room temperature, this protocol uses *Lytechinus pictus*. Adult animals can be obtained from commercial sources (see Chapter “[Procuring animals and culturing of eggs and embryos](#)” by Adams et al., this volume) and shipped to regions where the species is not indigenous. *Strongylocentrotus purpuratus*, the species in which the increase of efflux transport was first observed (Hamdoun et al., 2004), can also be used but requires that gametes and embryos be chilled (optimally to 15 °C). If adapting this lab for other species, a pilot experiment is suggested to confirm that the lab will work, since the activation of transport is more difficult to observe in certain species, such as those with high amounts of interfering pigments in the egg.

**FIG. 1**

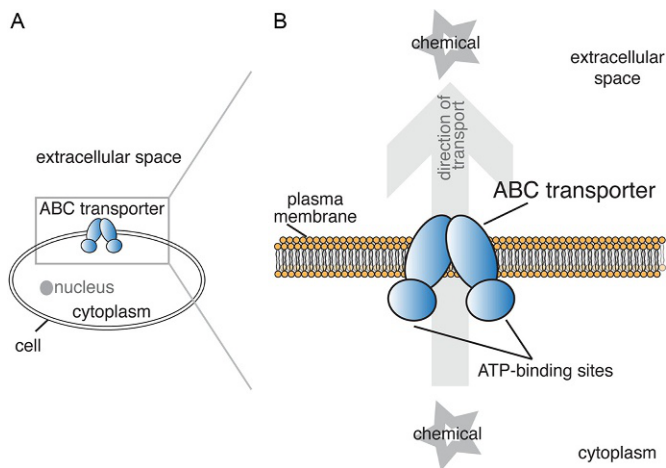
Lytechinus pictus eggs before (A) and after (B) sperm fusion. Fertilization is marked by the appearance of the fertilization envelope in (B). Eggs are 120 μm in diameter.

calcium levels within the egg, which is normally triggered by sperm-egg fusion. However, in the absence of living/healthy sperm, the appearance of the FE can also be aberrantly triggered by simply increasing the membrane's permeability to calcium (e.g., with calcium-ionophore or a detergent), thereby artificially increasing intracellular calcium and leading to activation of the egg.

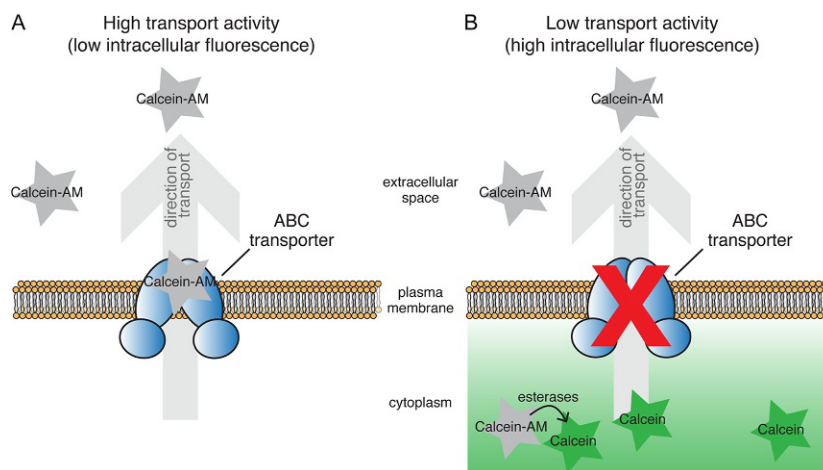
One mechanism embryos (and adult organisms) have evolved to protect against chemicals involve a group of transmembrane proteins called ATP-binding cassette (ABC) transporters (Fig. 2). ABC transporters use ATP to power transport of myriad substances across membranes (Fig. 2B), and some of these proteins specialize in the efflux of harmful chemicals from cells.

To monitor cellular accumulation or efflux of xenobiotics, it is necessary to have a reliable assay for detecting these chemicals. Assays that employ colored or fluorescent "reporter molecules" are especially useful, as we can detect their accumulation with a microscope. One such compound is Calcein-acetoxymethylester (calcein-AM, C-AM), a membrane permeable, non-fluorescent substrate of ABC transporters (Homolya, Hollo, Müller, Mechetner, & Sarkadi, 1996). Upon entering a cell (such as a sea urchin egg), C-AM is hydrolyzed to calcein by intracellular esterases, which causes the calcein molecule to fluoresce and lose its membrane permeability, thus trapping it within the cell. The only way the molecule can leave the cell is via transporter proteins (Fig. 3). Thus, when a cell has *high* ABC transport activity, intracellular fluorescence is *low* because the calcein is exported from the cell (Fig. 3A). When ABC transport activity is *low*, intracellular fluorescence is *high* because the calcein accumulates in the cell (Fig. 3B). We can measure transport activity by comparing fluorescence of different cells exposed to C-AM for the same period of time.

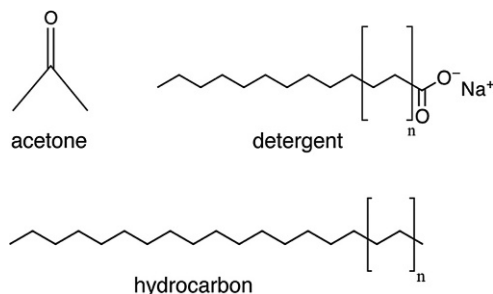
In today's laboratory, we will use gametes and embryos of the painted white sea urchin, *L. pictus*, to monitor cellular protective mechanisms and assess toxicity of several common chemicals/pollutants. To monitor protective mechanisms, we

**FIG. 2**

ABC transporters are found in the membranes of all known organisms. Members of this large family of proteins all use power released by the hydrolysis of ATP to transport substrates, often against their concentration gradients. Individual ABC transporters differ in the substrates they transport; some translocate lipids, others move ions, and still others transport signaling molecules. The subset of ABC transporters called Multidrug resistance (MDR) transporters can efflux xenobiotics from cells. They earned the “MDR” name because of their ability to keep chemotherapeutic drugs out of cancer cells, leading to multidrug resistance. (A) A schematic of a cell expressing an ABC transporter localized to the plasma membrane. (B, inset) ABC transporters use energy from ATP to efflux a variety of xenobiotics from the cytoplasm to the extracellular space.

**FIG. 3**

A schematic showing the expected results of the calcein-AM assay under conditions of high (A) and low (B) ABC transporter activity.

**FIG. 4**

Structures of acetone, detergent (soap), and hydrocarbons (motor oil). The unique molecular features of these chemicals render them toxic to living cells. Acetone in high quantities will dissolve cell membranes, altering their permeability to ions and other molecules and disrupting the cell's protective barrier. Additionally, due to its small size, acetone can enter the cytoplasm of the cell and interfere with enzymes that regulate cell metabolism. Detergents are water-soluble salts of fatty acids that are highly effective at permeating and disrupting cell membranes due to their ability to form micelles, which are single, spherical layers of detergent molecules that can surround membrane lipids in an aqueous environment. Hydrocarbons lack the charged head of a detergent and therefore cannot form micelles, but they can still enter the lipid bilayer and interfere with the function of proteins and lipids in the cell membrane.

will assess ABC transporter-mediated chemical efflux activity before and after fertilization, using the calcein accumulation assay described above and fluorescence microscopy. We will then run a modified version of the EPA toxicity test to evaluate sensitivity of marine organisms to several types of chemicals that humans commonly discharge into the environment (Fig. 4): organic solvents (acetone), detergents (household soap), and hydrocarbons (motor oil).

2.2 SPECIFIC ACTIVITIES AND LEARNING GOALS

Part A

Specific Activities: Fertilize eggs, and incubate eggs and embryos in a xenobiotic transporter indicator (Calcein-AM). Examine eggs and embryos on the microscope and collect data using fluorescence microscopy.

Learning goals: To practice basic laboratory skills including pipetting, calculating chemical dilutions, and formulating and testing a hypothesis. To collect and organize digital data from quantitative microscopy.

Part B

Specific Activities: Evaluate the toxicity of acetone, soap, and motor oil on marine embryos by running a modified version of the EPA fertilization toxicity test.

Learning goals: To critically evaluate the strengths and limitations of a protocol, and use these conclusions to optimize a protocol.

2.3 MATERIALS^b

- 0.22 μm filtered seawater (FSW) or artificial filtered seawater (AFSW)
- *L. pictus* eggs and sperm (50 μL eggs in 2.5 mL FSW; concentrated “dry” sperm on ice)
- Fertilized (0.5–1 h post-fertilization, hpf) *L. pictus* embryos
- *Chemicals*: acetone, household soap (hand/dish/laundry—avoid hand soap with added moisturizers), motor oil
- Calcein-AM (1 mM stock in DMSO)
- Plastic transfer pipettes
- P10, P200, P1000 pipettes and tips
- 1.5-mL microcentrifuge tubes
- Centrifuge (optional but preferred—to spin down C-AM dilution)
- Permanent markers
- Disposable round-bottom glass culture tubes, 13 \times 100 mm
- Microscope slides
- Compound microscopes with 10 \times and 20 \times objectives
- Fluorescence microscope (to be operated by instructor)

2.4 PART A: VISUALIZING EMBRYONIC PROTECTION USING A CHEMICAL EFFLUX ASSAY AND FLUORESCENCE MICROSCOPY

2.4.1 Procedure

1. From your instructor, obtain unfertilized eggs, 0.5–1 hpf fertilized eggs (embryos), and 1 mM calcein-AM (C-AM).
*Eggs and embryos have been prepared in 2.5 mL FSW.
 Avoid getting C-AM on your hands, as it is dissolved in DMSO, which carries solutes across cell membranes.*
2. Prepare the C-AM for 250 nM treatment.
 - a. Calculate what volume of 1 mM C-AM stock must be added to a final volume of 2.5 mL FSW for a final concentration of 250 nM C-AM: _____
Hint: $C_1V_1 = C_2V_2$
 - b. Do we have a pipette to transfer this volume of C-AM?
 - c. Make a 10 \times dilution of C-AM by adding 4 μL of 1 mM C-AM to 36 μL FSW in a microcentrifuge tube. Flick the tube vigorously to mix. *The C-AM stock solution is viscous, so be sure to mix well.* Collect the liquid at the tube bottom using the centrifuge. What concentration is your new stock? _____

^bNotes on preparing materials: For information about making artificial seawater (if filtered seawater is unavailable), obtaining sea urchin gametes, and starting embryo cultures, see Chapter “[Procuring animals and culturing of eggs and embryos](#)” by Adams et al. of this volume. To obtain the 1 mM Calcein-AM stock, a pre-made solution (1 mg/mL in DMSO, 1.006 mM) can be purchased (Thermo Fisher C3099). Alternatively, solid Calcein-AM (Thermo Fisher C1430) can be purchased, then resuspended with fresh DMSO and mixed well immediately before using.

3. Treat the eggs and embryos with C-AM.
 - a. Add your 6.25 μL of 0.1 mM C-AM to each glass tube of eggs/embryos. Gently invert to mix well. Mark the time of C-AM addition in your lab notebook.
4. Incubate samples in C-AM for 1.5–2 h on the benchtop.
5. Before continuing, formulate a hypothesis about which samples—eggs or embryos—will accumulate more calcein. Think about which developmental stage might activate stronger cellular protective mechanisms.

Write your hypothesis here, and explain your reasoning.
6. Continue to Part B while you are waiting for the incubation to finish, then return to measure calcein accumulation once you complete Part B^c.
7. Continue here after 1.5–2 h incubation and once Part B is completed.
8. Measure accumulation of calcein using the fluorescence microscope. (The instructor will operate the fluorescence microscope).
 - a. Prepare a slide with a drop of eggs, and bring it to your instructor.
 - b. Your instructor will visualize the eggs using a 10 \times or 20 \times objective and bright-field microscopy. When the eggs are in focus, the white light will be turned off, and fluorescence will be visible when calcein is excited with ~ 488 nm light.

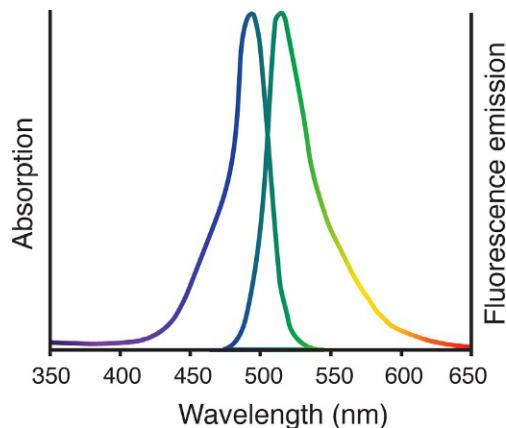
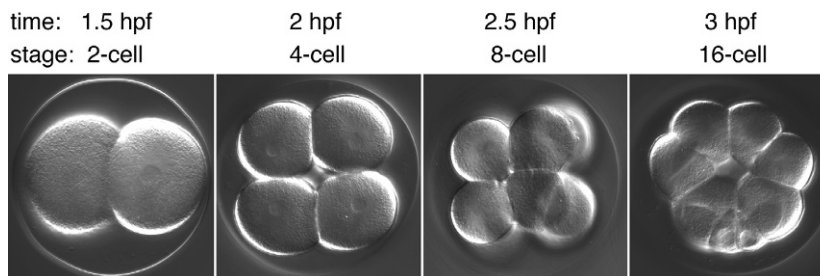


FIG. 5

The absorption and emission spectra of calcein.

^cThe excitation/emission maxima for calcein are 495 nm/515 nm (Fig. 5). Collect only the calcein fluorescence images; do not collect other channels such as brightfield or DIC, because it could potentially confuse students when they do the analysis in ImageJ. It is likely that the eggs will be at the maximum of the dynamic range, and the embryos will be at the minimum. Imaging settings (laser/lamp intensity, gain) should be based on egg fluorescence, as it will be much brighter than embryo fluorescence. If the dynamic range is too broad for the imaging setup, the concentration of calcein-am can be reduced accordingly.

**FIG. 6**

Development of sea urchin embryos over time. Micrographs show the progressive cell divisions that occur from 1.5 to 3 h post-fertilization (hpf) when embryos are grown at room temperature (22°C). Development will proceed faster if room temperature is higher and slower if room temperature is lower.

- c. Your instructor will capture images of >5 fluorescing eggs.
- d. Prepare a slide with a drop of embryos (their current developmental stage will depend on how long it has been since fertilization, Fig. 6), and bring it to your instructor to repeat imaging.

Make sure you obtain images of at least five eggs and five embryos.

9. The data (images) you collected will be analyzed during the next laboratory. Follow good practices when creating file names (e.g., YYYYMMDD_xenobioticlab_eggORembryo_sample#.xxx), and do not misplace the files.

Note: When organizing data files for an experiment, it is very important to have a logical and consistent file naming system. This is increasingly necessary as you generate larger data sets. For more suggestions on developing your own naming system, see:

<https://library.stanford.edu/research/data-management-services/data-best-practices/best-practices-file-naming>

2.4.2 Questions

1. Why is it important that C-AM is membrane permeable but calcein is not?
2. Why is it especially useful that calcein fluoresces, but C-AM does not?
3. How could we modify our experiment to measure chemical accumulation of a compound that is always fluorescent (whether it is inside the cell or not)?
4. Why is it necessary to compare samples that have been exposed to calcein for the same length of time? *Hint: Within the timeframe of this experiment, calcein accumulates linearly with time.*
5. Does it seem like all the eggs or embryos within your samples are equally fluorescent? What could account for any variation you see?
6. What would happen to ABC-transporter activity if a chemical depleted the cell's ATP? Now, ignoring the ABC-transporters in the cell, what else do you think would happen to the cell if its ATP were depleted?

2.5 PART B: THE SEA URCHIN FERTILIZATION EPA TOXICITY TEST

2.5.1 Procedure

Note: the actual EPA test involves soaking sperm in the test solution for 20 min, then adding eggs, waiting 20 more minutes, and finally counting >100 eggs to evaluate fertilization. We will be soaking both the sperm and eggs in the test solution for only 10 min, then we will mix them and wait another 10 min before counting 20 eggs to evaluate fertilization.

1. Obtain eggs, sperm, FSW, acetone, hand/dish/laundry soap, and motor oil.
2. Prepare four solutions—Control (FSW only), Acetone, Soap, and Oil—in two glass culture tubes each (eight tubes total).
 - a. Label one tube “Control eggs” and another “Control sperm,” and fill them each with 3 mL FSW.
 - b. Label one tube “Acetone eggs” and another “Acetone sperm,” and fill them each with 3 mL FSW + 30 μ L acetone. Flick to mix.
 - c. Label one tube “Soap eggs” and another “Soap sperm,” and fill them each with 3 mL FSW + 5 μ L soap. Flick to mix.
 - d. Label one tube “Oil eggs” and another “Oil sperm,” and fill them each with 3 mL FSW + 5 μ L oil. Flick to mix.
3. Add gametes to each test solution. Mark the time of addition in your lab notebook.
 - a. To each “eggs” test solution, add 50 μ L settled eggs. Flick the tubes to mix thoroughly.
 - b. To each “sperm” test solution, add 3 μ L sperm. Sperm is viscous, so pipette very slowly to allow the sperm to travel through the pipette tip. Flick the tubes to mix thoroughly.
4. Wait 10 min, then fertilize the eggs. First flick the sperm solution tube to mix, then add 100 μ L to the appropriate tube of eggs.
 - a. Add 100 μ L “Control sperm” solution to the “Control eggs” tube, and mix by flicking. Record the time of sperm addition in your notebook.
 - b. Mix the Acetone eggs and sperm similarly, and record the time of sperm addition in your notebook.
 - c. Mix the Soap eggs and sperm similarly, and record the time of sperm addition in your notebook.
 - d. Mix the Oil eggs and sperm similarly, and record the time of sperm addition in your notebook.
5. Wait 10 min, then determine the percent of fertilized eggs in each test solution by counting how many eggs do and do not have a FE under the microscope. Begin with the controls (FSW only), which should have at least 70% fertilization.
 - a. Prepare a microscope slide with eggs. Flick the tube to resuspend the eggs in the water and use a plastic transfer pipette to transfer one drop onto the slide.
 - b. Use the 20 \times objective on your microscope to visualize the eggs in the droplet. Take care not to plunge the objective into the droplet while you are

focusing the microscope. We are not using coverslips, so this is important as seawater can ruin an objective!

- c. Count the first 20 eggs you see and record in your notebook how many of them are fertilized (i.e., have a clear fertilization envelope, FE; refer to Fig. 1).
6. Answer the following questions before continuing:
 - a. By measuring the presence of the FE, what could we be measuring other than just fertilization? *Hint: refer to the background information for this lab.*
 - b. How could we modify the protocol to ensure we are observing just fertilization? Come up with a quick experiment and try it for each of the chemicals that produced “fertilization” before returning to Part A^d. Record your experimental design and results in your lab notebook.
7. Return to Part A to measure calcein accumulation in the eggs/embryos. Continue here when you complete Part A.
8. Compare the egg fertilization percentages in your acetone, soap, and oil solutions to the control solution.
 - a. Draw a bar graph of the fertilization percentages. Plot “% fertilized” on the *y*-axis, and your various treatments (control, acetone, soap, and oil) on the *x*-axis.

2.5.2 Questions

1. How do the fertilization percentages change in response to acetone, soap, and motor oil?
2. What conclusions can you draw about the toxicity of each chemical to sea urchin gametes?
3. Do you think an activated egg (as opposed to a fertilized egg) will develop into a healthy sea urchin? Discuss the genetic basis of your reasoning. *Hint: Think about ploidy.*
4. Do you think the EPA fertilization test is an appropriate way to measure toxicity? Why or why not?

3 LABORATORY DAY 2: ANALYZING AND PRESENTING SCIENTIFIC DATA

3.1 BACKGROUND

In the previous laboratory, we performed a calcein accumulation assay with unfertilized eggs and embryos to assess ABC transporter-mediated efflux activity. We then performed a modified version of the EPA toxicity test, using fertilization of sea urchin eggs as an endpoint, to assess toxicity of acetone, soap, and motor oil.

^dStudents will hopefully propose that they expose eggs to acetone, soap, and oil, then evaluate them without adding sperm. This will resolve whether the FE is the result of fertilization or activation. Anticipated results: 30 μ L acetone will fertilize (but they will look unhealthy), 5 μ L soap will activate not fertilize, and 5 μ L motor oil will neither activate nor fertilize.

Today, we will analyze the data collected in the previous lab using ImageJ free-ware made available by the National Institutes of Health (NIH). We will quantify the calcein fluorescence in unfertilized eggs and 2–8 cell embryos, then compare the ABC transporter efflux activity of eggs and embryos based on our data. We will use Microsoft Excel to plot data from both the calcein assay and the modified EPA assay, and we will perform basic descriptive statistics with the calcein assay data to determine if our findings are significant.

3.2 SPECIFIC ACTIVITIES AND LEARNING GOALS

Specific Activities: Collect data from fluorescence microscope images, graph quantitative fluorescence data, and perform descriptive statistics.

Learning Goals: To learn techniques and practices for quantitative image analysis and gain an introduction to common types of scientific software including ImageJ and Excel. To test hypotheses generated on Day 1.

3.3 MATERIALS

- Calcein fluorescence images collected during the previous lab
- Computer
- ImageJ64 freeware
- Data collected during previous lab
- Microsoft Excel (or comparable freeware)

3.4 USING IMAGEJ TO ANALYZE EFFLUX ACTIVITY DATA COLLECTED ON DAY 1

3.4.1 Procedure

1. Download ImageJ onto your computer by following the instructions at: <https://imagej.nih.gov/ij/download.html>
 - a. For more information on ImageJ, a general tutorial can be found here: <https://imagej.nih.gov/ij/docs/guide/user-guide.pdf>. However, the below instructions should be sufficient for completing today's analyses.
2. Open the calcein image files collected during the previous laboratory in ImageJ.
 - a. If the image opens and you cannot see the eggs/embryos, click on your image, then go to **Image > Adjust > Brightness/Contrast**. A window will appear where you can adjust brightness/contrast (Fig. 7A). If the eggs/embryos are too dim to see, it is important to increase the brightness enough that you can see the outline of the cells (Fig. 7B). Adjusting the brightness here will not affect your fluorescence measurements (the program retains the original numerical brightness data regardless of how you visually manipulate the image here).

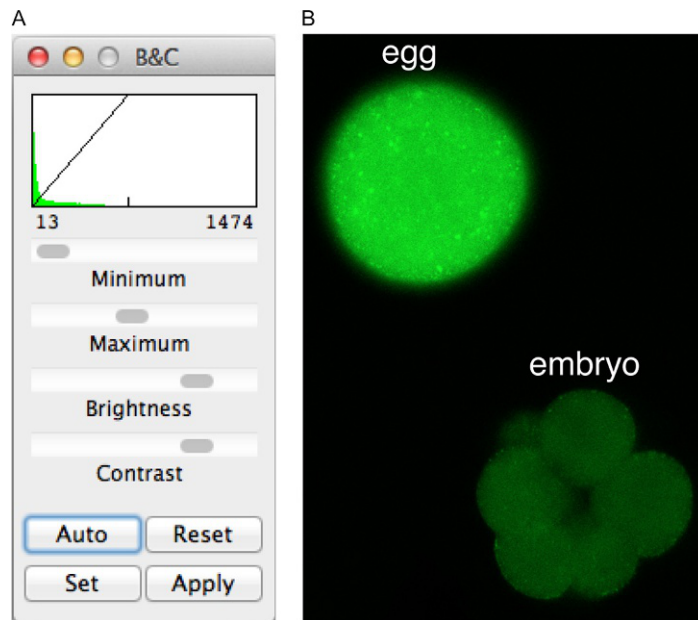
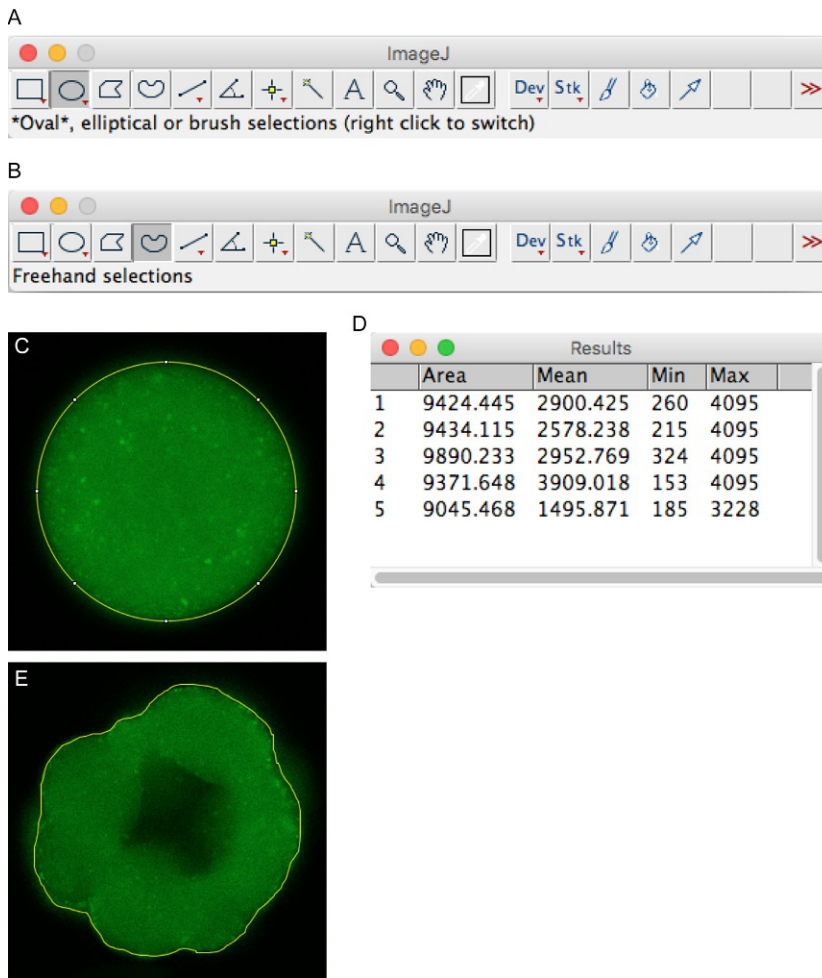


FIG. 7

How to adjust image brightness in ImageJ. (A) The Brightness/Contrast control panel. (B) A calcein-exposed egg and embryo of sufficient brightness to see and outline.

3. Quantify the calcein fluorescence of five eggs and five embryos. Begin with the eggs.
 - a. Locate the toolbar, and use either the **oval tool** (Fig. 8A) or the **freehand tool** (Fig. 8B) to draw around the outline of your egg (Fig. 8C).
 - i. If you use the **oval tool**, you can hold down **shift** as you are drawing to make it a circle. Once you have drawn the circle, you can click in the center of your circle and drag it if it needs to be moved slightly. You can also adjust the size/dimensions by dragging the small white boxes distributed along the yellow outline.
 - ii. If you use the **freehand tool**, you cannot move the shape once it has been drawn.
 - b. Measure the fluorescence inside the outline you drew, called the Region Of Interest (ROI), by clicking on **Analyze > Measure**. A “Results” window will appear, listing the Area of your ROI, the Mean fluorescence of your ROI, and the Minimum and Maximum fluorescence of your ROI (Fig. 8D).
 - c. Draw an outline of your next egg, repeating the same process as described above, and again go to **Analyze > Measure**. A new row will be created with the data from this second ROI. If you need to open a new image to locate another egg, this is not a problem; each measurement is added as a new line regardless of which image file it came from.

**FIG. 8**

How to outline eggs and embryos to measure fluorescence in ImageJ. (A) The location of the Oval tool. (B) The location of the Freehand tool. (C) A calcein-exposed egg outlined for analysis using the Oval tool. (D) The Mean column in the Results panel shows calcein fluorescence measurements of five different samples. (E) A calcein-exposed embryo outlined for analysis using the Freehand tool.

- d. Repeat this procedure until you have five eggs measured.
- e. After you measure five eggs and have five rows of data, click on the “Results” window, then go to **File > Save As**, name the file “YYYYMMDD_xenobioticlab_egg_results”, and save your data as a type.xls file.
- f. Repeat the measurements with your embryo samples. If they are 2-cell stage or older, you will need to use the **freehand tool** (Fig. 8B) to outline the embryos (Fig. 8E).

- g. After you measure five embryos, save your results as “YYYYMMDD_xenobioticlab_embryo_results.xls”, as you did for the egg data.
4. Graph your calcein fluorescence values as a bar graph in Microsoft Excel, OpenOffice, or comparable software.
- These instructions were written for use with Microsoft Excel. Options and formulas may be slightly different depending on which version of Excel (Mac, PC, year) is used. The procedure to graph data should be similar in other programs, and in most cases a simple Google search can resolve any discrepancies.*
- a. In Excel, open your results files (“YYYYMMDD_xenobioticlab_egg_results.xls” and “YYYYMMDD_xenobioticlab_embryo_results.xls”).
- b. Combine all the data into one document: copy data from each set of results and paste it into a new Excel document. Label the two groups of results “Egg” and “Embryo.” Name this new document appropriately using your consistent file naming system.
- c. We will work only with the **mean fluorescence** values (which should be in column C) and will ignore the Area, Min, and Max values.
- d. For each group of data points (egg and embryo), take your five mean fluorescence values and calculate the **average**. To do this, click on the cell below the fifth mean fluorescence value. Type “=average(”, then use the mouse to highlight all five mean fluorescence values, type “)”, and hit **Enter/Return** on your keyboard. The program will calculate the average of the five values you selected.
- e. Now calculate the **standard deviation** for each average. Click on the cell below the one containing the average calculation. Type “=stdev(”, then use the mouse to highlight all five mean fluorescence values, type “)”, and hit **Enter/Return** on your keyboard. The program will calculate the standard deviation of the five values you selected around the average.
- f. In the cells to the left of your calculated average and standard deviation values, label them “Egg average fluorescence,” “Egg standard deviation,” “Embryo average fluorescence,” and “Embryo standard deviation.” You will plot the Egg average fluorescence and Embryo average fluorescence in a bar graph, and you will represent each group’s standard deviation with error bars.
- g. In a blank area of the spreadsheet, rearrange your calculated values so they are in the order shown in Fig. 9. Do not simply copy and paste, because the program will paste the formula rather than the numbers. Instead, to paste the average and standard deviation values, use **Edit > Paste Special**. Under “Paste”, select **Values**. Under “Operation”, select **None**. Click **OK**.
- h. Create a bar graph of your average fluorescence values. Highlight the four cells shown in dark gray (Fig. 9; Egg, ###, Embryo, ###). In the menu, click on **Charts**, then **Column**, and under 2-D column, click **Clustered**

	Average fluorescence	Standard deviation
Egg	###	###
Embryo	###	###

FIG. 9

How to arrange calculated values in Excel to generate a bar graph with error bars.

- Column.** Labels will automatically be added to the x -axis. Delete the legend, as it is unnecessary.
- i. Create error bars representing the standard deviation around each average. In the graph, double click on the egg bar. A “Format Data Series” window will open. On the left panel of this window, click **Error Bars**. Under “Display,” click **Both**. Under “End style,” click **Cap**. Under “Error amount,” click **Custom**, then click **Specify Value**. A “Custom Error Bars” window will open. In the “Positive Error Value” space, highlight and delete anything in the space, then highlight the two standard deviation values shown in light gray (Fig. 9). This will create positive error bars the length of your calculated standard deviation values. Repeat the same process in the “Negative Error Value” space. Click **OK**. Click **OK** again.
 - j. Complete your graph with axis labels and a figure legend. Label the appropriate axis “Mean fluorescence (arbitrary units)” and the other axis “Developmental stage of *L. pictus*.” Print this graph to include in your lab notebook and write a descriptive figure legend^e
5. To test whether the difference in calcein accumulation you measured in eggs versus embryos is significant, perform a statistical analysis using a two-tailed unpaired Student’s t -test^f. We use the Student’s t -test because it is the most

^eAnticipated results: eggs will accumulate significantly more calcein than embryos, indicating that ABC transport activity is higher in embryos than unfertilized eggs.

^fDepending on the version of Microsoft Excel used by the students, it may be necessary to provide alternative instructions to perform the analysis. Here are instructions to install and use StatPlus, which will enable descriptive statistics for Excel for Mac 2011 users: After you have entered all the data into Excel, Download StatPlus from: <http://www.analystsoft.com/en/products/statplusmac/>

1. Start the STATPLUS program. Excel should open.
2. Click on the StatPlus icon. Go to STATISTICS > BASIC STATISTICS AND TABLES > DESCRIPTIVE STATISTICS. A Descriptive Statistics window will display. Place the cursor in the Variables Box.
3. Return to the Excel worksheet and select the cells containing the data. Return to the Descriptive Statistics window, where the cells and worksheet will be referenced in the Variables Box.
4. Find the “Labels in First Row” option in the lower left corner of the Descriptive Statistics window and determine whether the box is checked. If the first row selected contains data, uncheck the box or else those data will not be included in the calculation.
5. Click OK. Allow StatPlus is performing the analysis without returning to Excel. A new worksheet will open showing the descriptive statistics once StatPlus has finished. Copy and paste the results into the worksheet with the data.

commonly used technique for testing whether two datasets are different based on the difference in means between two samples.

- a. On the same Excel worksheet containing your egg and embryo fluorescence data, in an empty cell, type “=ttest(”, then use the mouse to highlight all five mean egg fluorescence values, type “,”, then use the mouse to highlight all five mean embryo fluorescence values, type “,”, type “2,”, type “2)”. Press **Enter/Return**.
 - b. The cell you typed in will now display the P -value for the t -test. Record your P -value in your lab notebook. You may also write it on the graph you generated in Step 4 and/or include it in your descriptive figure legend. Typically, a P -value of <0.05 is considered “statistically significant.” This is because the P -value denotes the probability of obtaining results as or more extreme than the observed results, assuming there is *no* difference between the two conditions (e.g., $P = 0.05$ means 5% probability). Therefore, the smaller the P -value, the less likely it is that as or more extreme results could be obtained, and so with a P -value below our pre-determined threshold of 0.05, we reject the hypothesis that there is no difference between the two conditions, and accept the hypothesis that the difference is statistically significant.
 - c. For a more in-depth overview of the t -test, see below link: <http://www.chem.utoronto.ca/coursenotes/analsci/stats/ttest.html>
6. Now that you have some familiarity with generating bar graphs in Excel, transfer your data from the modified EPA toxicity test into an Excel spreadsheet, and create a bar graph (you will not have error bars on this graph). Write a descriptive figure legend.

3.4.2 Questions

1. Which had higher calcein accumulation: eggs or embryos?
2. What does this tell us about the relative ABC transporter efflux activity in eggs compared to embryos?
3. Do you think eggs or embryos are more “protected” from chemicals in the environment? Explain how your experimental results led you to draw this conclusion.
4. If you were to perform the EPA toxicity test from the previous lab—but this time you fertilized the eggs first, waited 1 h, then exposed them to the test chemicals—do you think the embryos would be more or less affected by the chemicals? Why?

REFERENCES

- Dinnel, P. A., Link, J. M., Stober, Q. J., Letourneau, M. W., & Roberts, W. E. (1989). Comparative sensitivity of sea urchin sperm bioassays to metals and pesticides. *Archives of Environmental Contamination and Toxicology*, 18(5), 748–755. <https://doi.org/10.1007/BF01225012>.

- Epel, D. (1972). Activation of an Na⁺-dependent amino-acid transport-system upon fertilization of sea-urchin eggs. *Experimental Cell Research*, 72(1), 74. [https://doi.org/10.1016/0014-4827\(72\)90569-1](https://doi.org/10.1016/0014-4827(72)90569-1).
- Epel, D. (1975). The program of and mechanisms of fertilization in the echinoderm egg. *American Zoologist*, 15(3), 507–522.
- Epel, D., Vacquier, V. D., Peeler, M., Miller, P., & Patton, C. (2004). Sea urchin gametes in the teaching laboratory: Good experiments and good experiences. *Methods in Cell Biology*, 74, 797–823.
- Giacomini, K., Huang, S., Tweedie, D., Benet, L., Brouwer, K., Chu, X., et al. (2010). Membrane transporters in drug development. *Nature Reviews Drug Discovery*, 9(3), 215–236.
- Gottesman, M. M., Fojo, T., & Bates, S. E. (2002). Multidrug resistance in cancer: Role of ATP-dependent transporters. *Nature Reviews Cancer*, 2(1), 48–58. <https://doi.org/10.1038/nrc706>.
- Hamdoun, A. M., Cherr, G. N., Roepke, T. A., & Epel, D. (2004). Activation of multidrug efflux transporter activity at fertilization in sea urchin embryos (*Strongylocentrotus purpuratus*). *Developmental Biology*, 276(2), 452–462. <https://doi.org/10.1016/j.ydbio.2004.09.013>.
- Homolya, L., Hollo, M., Müller, M., Mechetner, E. B., & Sarkadi, B. (1996). A new method for quantitative assessment of P-glycoprotein-related multidrug resistance in tumour cells. *British Journal of Cancer*, 73(7), 849. <https://doi.org/10.1038/bjc.1996.151>.
- Matese, J. C., Black, S., & McClay, D. R. (1997). Regulated exocytosis and sequential construction of the extracellular matrix surrounding the sea urchin zygote. *Developmental Biology*, 186(1), 16–26. <https://doi.org/10.1006/dbio.1997.8585>.
- Pearse, J. S., & Cameron, R. A. (1991). Echinodermata: Echinoidea. In *Vol. 6. Reproduction of marine invertebrates: Pacific Grove: Reproduction of Marine Invertebrates*.
- Piatigorsky, J., & Whiteley, A. H. (1965). A change in permeability and uptake of [14C] uridine in response to fertilization in *Strongylocentrotus purpuratus* eggs. *Biochimica Et Biophysica Acta (BBA)—Nucleic Acids and Protein Synthesis*, 108(3), 404–418. [https://doi.org/10.1016/0005-2787\(65\)90033-X](https://doi.org/10.1016/0005-2787(65)90033-X).
- Schindelin, J., Rueden, C. T., Hiner, M. C., & Eliceiri, K. W. (2015). The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular Reproduction and Development*, 82(7–8), 518–529. <https://doi.org/10.1002/mrd.22489>.
- U.S. Environmental Protection Agency. (2002). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. In *Saltwater chronic methods manual* (3rd ed.). Cincinnati, OH: Office of Water EPA-821-R-02-014.
- U.S. Environmental Protection Agency. (2009). Sperm cell toxicity tests using the sea urchin (*Arbacia punctulata*). In *Supplement to training video, Whole effluent toxicity training video series: Saltwater series*. EPA 833-C-09-001.
- Vacquier, V. D. (2011). Laboratory on sea urchin fertilization. *Molecular Reproduction and Development*, 78(8), 553–564. <https://doi.org/10.1002/mrd.21360>.
- Whiteley, A. H., & Chambers, E. L. (1966). Phosphate transport in fertilized sea urchin eggs. II. Effects of metabolic inhibitors and studies on differentiation. *Journal of Cellular Physiology*, 68(3), 309–323. <https://doi.org/10.1002/jcp.1040680311>.